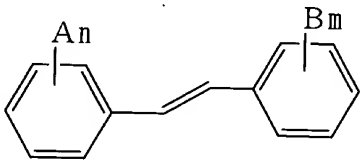
Appendix A

ABSTRACT

The present invention provides a composition for preventing or treating diseases accompanied by a decrease in bone weight, hypertension and diseases resulting from hypertension, the composition containing, as an active component, at least one member selected from a compound represented by formula (1)



(1)

[wherein A and B are the same or different and each represents a halogen atom, an amino group, an amidino group, an anilinoamide group, a mercapto group, a sulfonic acid group, a phosphate group, a carboxy group, a hydroxy C_1 to C_5 alkyl group, a sugar residue, $-OR^1$ (R^1 represents a hydrogen atom, a C_1 to C_5 alkyl group, a hydroxy C_1 to C_5 alkyl group or a C_2 to C_5 alkenyl group.) or $-OCOR^2$ (R^2 represents a C_1 to C_5 alkyl group, a hydroxy C_1 to C_5 alkyl group or a C_2 to C_5 alkenyl group.).

n and m are the same or different and each is an integer from 0 to 5. There are n A's and m B's each of which may be the same or different.] and its multimers.

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Appendix B

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Age and sex dependency of the biochemical indices of bone remodelling

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Key words: Age; Puberty; Menopause; Bone enzymes; Bone remodelling; Isoenzymes; Alkaline phosphatase; Acid phosphatase; Hydroxyproline

Summary

The values for the bone isoenzyme of serum alkaline phosphatase peak in the first two years of age, between 6 and 7 years of age, before the end of puberty and in the postmenopause. A population between the ages of 29 and 45 provides a reference population to which all other age groupings can be compared. A significant positive correlation was found between bone isoenzyme of serum alkaline phosphatase and urinary hydroxyproline excretion in children as well as after puberty. However, in the children the urinary hydroxyproline excretion was significantly higher when compared with the bone isoenzyme of alkaline phosphatase. A significant positive correlation was found between the bone isoenzyme of alkaline phosphatase and plasma tartrate-resistant acid phosphatase, irrespective of age and sex. The biochemical indices of bone remodelling correlated significantly with the growth rate in children and adolescents. The results are in good agreement with the concept of the coupling of bone formation to bone resorption.

Introduction

Skeletal remodelling by osteoclasts and osteoblasts refers to the process of bone resorption and formation, respectively. These two bone processes are coupled not only in healthy adults [1] but also in some cases of abnormal bone metabolism, e.g.

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hyperparathyroidism [2]. In some diseases, e.g. multiple myeloma, a dissociation is found between bone resorption and bone formation [3]. Osteoblastic function is reflected by the activity of circulating bone isoenzyme of alkaline phosphatase (orthophosphoric–monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1, ALP) [4]. Bone remodelling by osteoclasts is reflected by urinary hydroxyproline excretion [5] and by plasma tartrate-resistant acid phosphatase (orthophosphoric–monoester phosphohydrolase (acid optimum), EC 3.1.3.2, ACP) [2,6].

In normal individuals, the urinary hydroxyproline excretion and the activity of bone isoenzyme of serum ALP and plasma tartrate-resistant ACP have been found to vary with age [6–9], increasing during periods of rapid growth. This prompted us to compare the relationships between the biochemical indices of bone remodelling during childhood, adolescence and in healthy adults.

Materials and methods

Population

The biological variations of the bone isoenzyme of serum ALP were determined in a population sample of 2100 subjects (947 males and 1153 females) who came for a health check-up between 1978 and 1983. Health criteria for acceptance into the study were: an absence of familial diseases in parents and siblings, no past or present history of hepatobiliary, renal or skeletal disorder (X-ray examination) or diseases likely to be associated with skeletal wasting, such as thyrotoxicosis, diabetes, hypercorticism, malabsorption, rheumatoid arthritis and other crippling diseases. Also excluded were pregnant women and individuals treated with sex hormones, thiazides, or other drugs known to influence calcium metabolism. The same criteria were applied to school children, students, and blood donors used in this study. 22% of the population were 1 to 14 year old children, and 39% were adults older than 45 years.

The activity of plasma tartrate-resistant ACP and urinary hydroxyproline excretion were determined on a more limited sample of 330 and 680 individuals, respectively, of whom 26% and 17%, respectively, were children 1 to 14 years old, and 32% and 43%, respectively, were adults older than 45 years.

The mean growth rate (cm/year) for the particular age and sex in children and adolescents was taken from an anthropometric study performed by the Institute of Hygiene and Epidemiology, Prague, 1981. All subjects were within normal weight and height range according to age and sex, as determined by the table from the above study. All subjects were residents of the city of Prague.

Blood samples were taken between 07.00 and 08.00 from the cubital vein after a fasting period of approximately 12 h, with use of tourniquet for as short a time as possible. The serum was separated within 1 h of sampling.

Biochemical analysis

The activity of bone isoenzyme of serum ALP (bone ALP) was determined with 4-nitrophenyl phosphate as substrate using an inactivation-inhibition method [10]. One unit (U) of enzyme activity corresponds to the hydrolysis of 1 μmol of substrate per min. The interassay coefficient of variation was 5.6%.

The activity of plasma tartrate-resistant ACP (TR-ACP) was determined with 4-nitrophenyl phosphate as substrate within 10 min of sampling [2]. The interassay variation was 5.2%.

Total hydroxyproline in urine was measured by the method described by Dubovský et al. [11]. The interassay variation was 3.6%. All subjects were given a hydroxyproline-free diet for 48 h prior to urine collection and for another 8-h period during which the urine was collected.

The reference Z-score (RZ-score) is the calculated deviation from the mean value of the distribution of samples of the reference population expressed as standard deviation (SD). The population between the ages of 29 and 45 was taken as the reference one. The relationship between urinary hydroxyproline excretion and bone ALP (HBP) was expressed as a difference between the respective RZ-scores.

Statistical analysis

Conventional statistical methods were used for calculation of means, standard deviations, winsorized means with the shortest confidence intervals, percentiles and

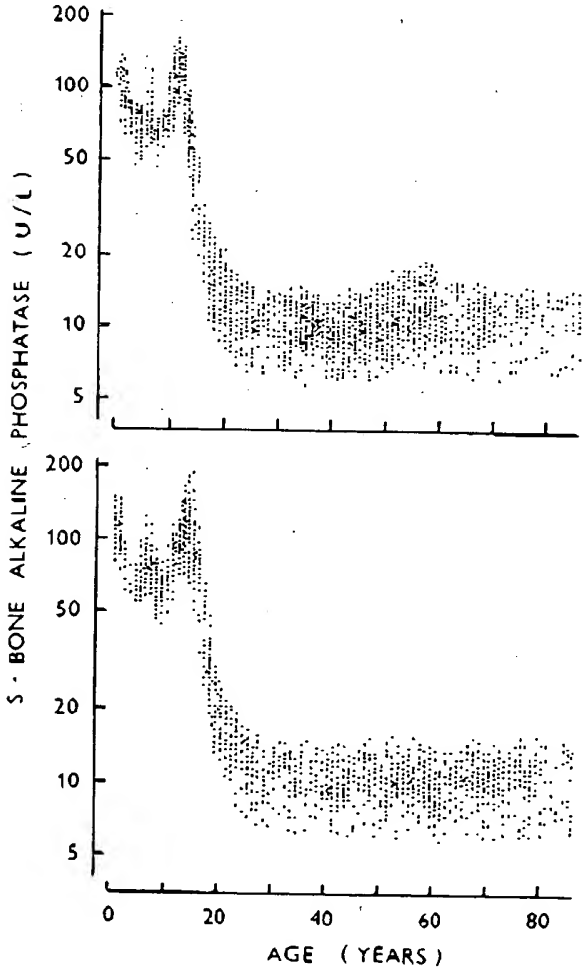

Fig. 1. Scattergrams of bone isoenzyme of serum ALP by age in healthy females (top) and males (bottom).

TABLE I

Bone isoenzyme of serum alkaline phosphatase in some age groups

Age group (years)	No. of cases males/ females	Males			Females		
		mean (U/l)	2 SD range (U/l)	RZ-score (mean)	mean (U/l)	2 SD range (U/l)	RZ-score (mean)
1– 2	48/46	100.6	68.0–148.7	10.6	90.9	61.4–134.7	10.4
3– 5	25/33	66.3	50.6– 86.9	8.6	67.4	48.0– 94.7	8.6
6– 7	32/25	83.4 [a]	57.3–121.4	9.6	71.2	44.7–113.3	9.4
8–12	74/40	75.0	45.7–123.2	9.2			
8–10					83.2	55.7–124.1	9.6
13–14	50/45	123.1 [a]	83.5–181.3	11.6			
11–12					109.4	69.4–172.3	11.0
21–25	53/77	13.5 [a]	7.9– 22.9	1.2	11.1	5.8– 21.2	0.4
29–45	189/267	10.3	6.7– 15.7	0	9.8	6.4– 15.0	0
51–60	127/170	10.0	6.6– 15.1	−0.2	12.0 [a]	7.2– 19.8	0.6
61–85	233/184	10.1	6.4– 16.1	0	10.8 [a]	6.8–117.2	0.2

[a] Probability by use of one-way analysis of variance, as compared with the other sex group, $p < 0.005$. Before puberty, the corresponding sex groups are compared.

regression analysis using BMDP 1V, 1R, and 7D programs of the University of California Health Computing Facility [12]. The statistical analysis was applied to logarithmically transformed data. The original skewed data were tested to confirm the validity of the transformation.

Results

The distribution of bone ALP with age in healthy individuals (Fig. 1) shows a steady decline in the enzyme activity towards the adult values during adolescence in both sexes. In male adolescents, however, it is more prolonged ($p < 0.001$). Between the ages of 24 and 45 in women, and 29 and 85 in men the mean bone ALP remains relatively unchanged (Table I).

In women, a significant bone ALP peak occurs between the ages of 50 and 60;

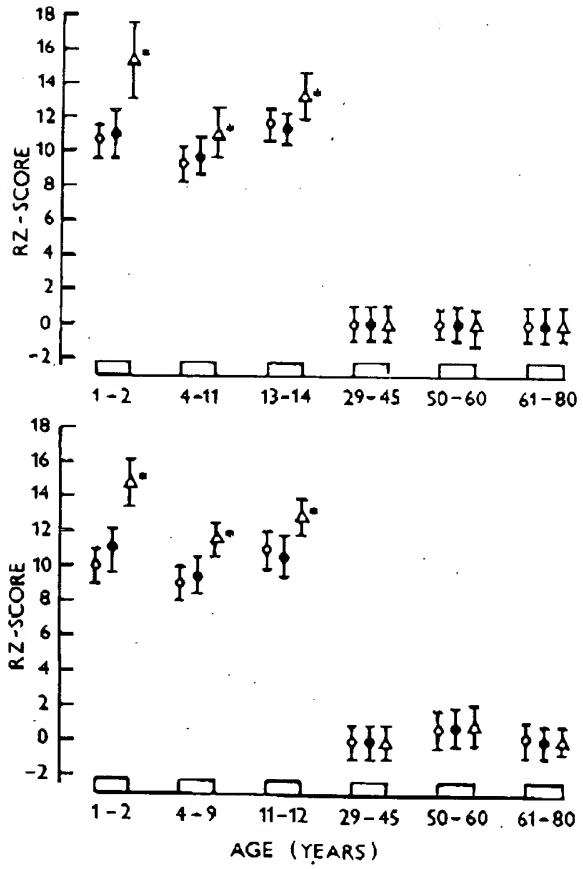

Fig. 2. Biochemical indices of bone remodelling by age in healthy males (top) and females (bottom). Reference population mean and SD (log transformation) for bone ALP, TR-ACP and urinary hydroxyproline excretion: 1.01 and 0.09, 1.21 and 0.07, and 0.62 and 0.05, respectively. ○: bone ALP (for the six age groups, $n = 48$, 111, 50, 189, 127 and 233, respectively, in males, and 46, 80, 45, 267, 170 and 184, respectively, in females). ●: TR-ACP (for the six age groups, $n = 6$, 25, 10, 36, 20 and 15, respectively, in males, and 6, 11, 10, 50, 50 and 13, respectively, in females). △: urinary hydroxyproline excretion (for the six age groups, $n = 10$, 28, 15, 78, 44 and 57, respectively, in males, and 6, 13, 11, 122, 101 and 56, respectively, in females). * probability by use of one-way analysis of variance, as compared with the other variables, $p < 0.005$.

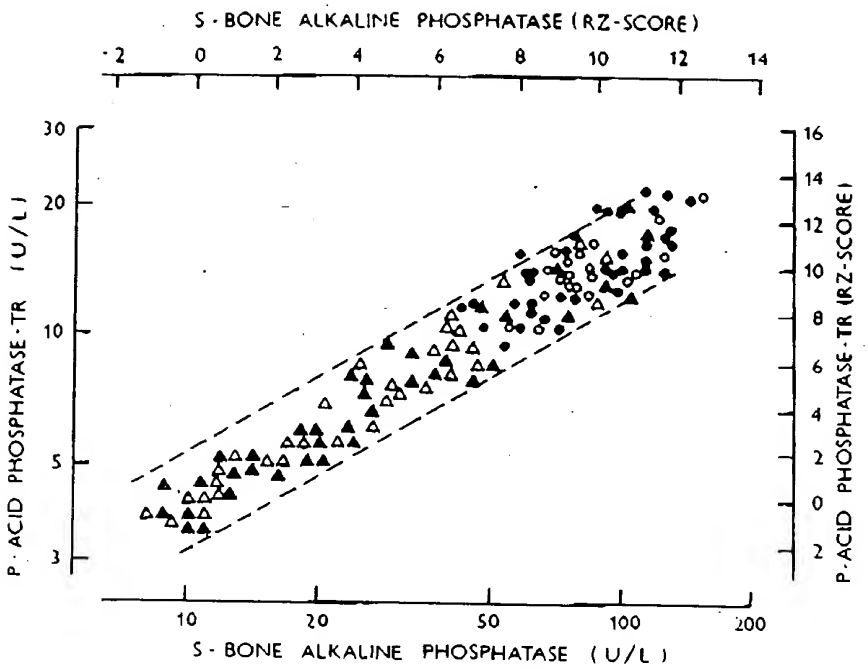

Fig. 3. Relationship between the activity of bone ALP and plasma tartrate-resistant ACP in children before the end of puberty (circles, $\log y = 0.46 \log x + 0.28$, $r = 0.71$, $n = 58$; $p < 0.001$) and from the end of puberty to 28 years of age (triangles, $\log y = 0.58 \log x + 0.03$, $r = 0.96$, $n = 70$, $p < 0.001$). Black, males; white, females. The dotted lines indicate the 95% confidence limits of the older group.

19% of the values are above the upper limit of the reference population. A sex-related difference was observed in the bone ALP in older adults as well (Table I).

The standard error of the mean activity of bone ALP did not change with age (SEM log bone ALP, mean ± SD, 0.06 ± 0.02 U/l, range 0.03–0.13 U/l, see also Fig. 4). Similar results were obtained with TR-ACP and urinary hydroxyproline. Therefore, it is possible to compare the different variables in different age groupings (Fig. 2).

A significant positive correlation was found between bone ALP and TR-ACP, irrespective of age and sex (Fig. 3). Accordingly, the distribution of TR-ACP with age fitted well with the distribution of bone ALP (Fig. 2).

The distribution with age and sex of urinary hydroxyproline excretion was similar to that of bone ALP with the exception of children and some women between the ages of 49 and 60 (Figs. 2 and 4).

A significant positive correlation was found between bone ALP and urinary hydroxyproline excretion in children as well as after puberty (at sexual maturation 5) (Fig. 5). However, these two regressions differ significantly ($p < 0.001$). Accordingly, the HBP values were significantly higher in children as compared with the group from the end of puberty to 28 years of age (2.6 ± 1.6 and 0 ± 0.98, respectively, $p < 0.001$). The HBP was not sex dependent. In the reference population, the relationship between the variables ($\log$ hydroxyproline $= 0.53 \log$ bone ALP $+ 0.67$, $n = 200$, $r = 0.72$, $p < 0.001$) was similar to that in the group from the end of puberty to 28 years of age.

Similarly, a significant positive correlation found between TR-ACP (x) and

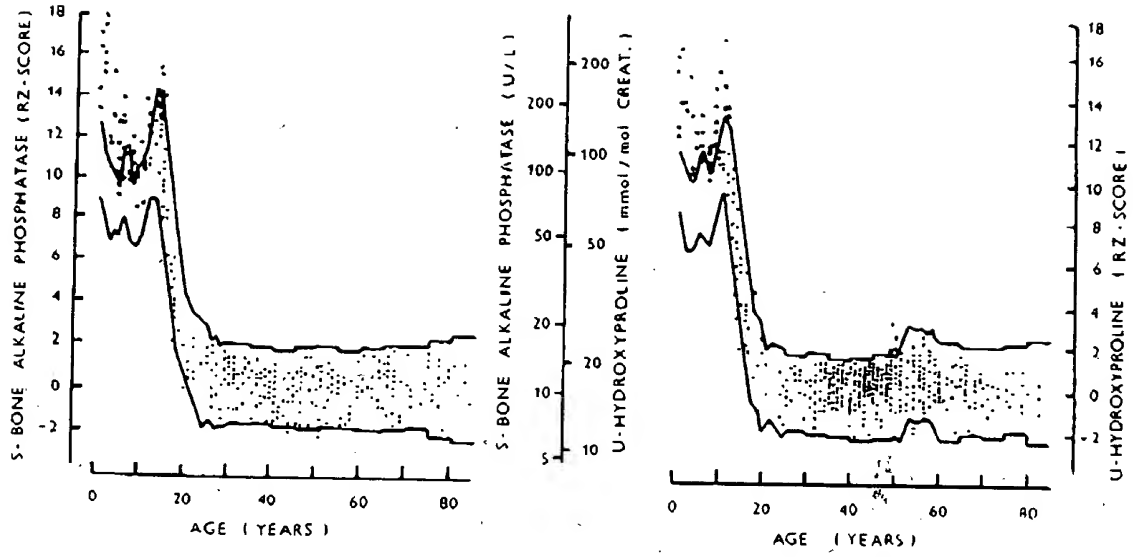

Fig. 4. Scattergram of urinary hydroxyproline excretion by age in healthy males (left) and females (right). The lines indicate P3 and P97 limits of the values for the activity of bone ALP.

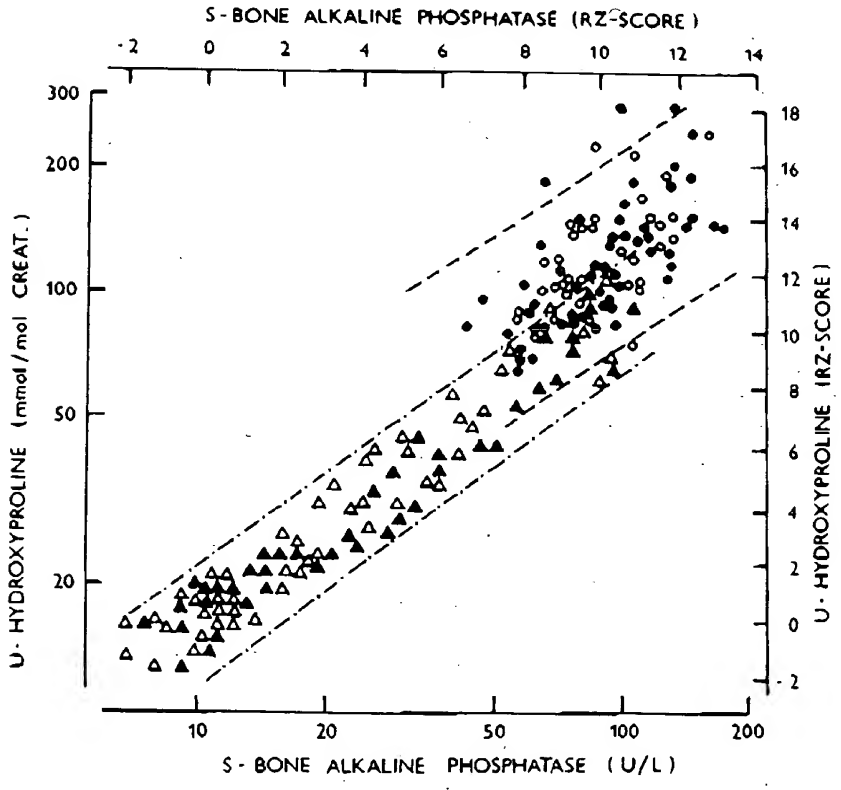

Fig. 5. Relationship between the activity of bone ALP and urinary hydroxyproline excretion in children before the end of puberty (circles, $\log y = 0.70 \log x + 0.71$, $r = 0.63$, $n = 93$, $p < 0.001$) and in individuals from the end of puberty to 28 years of age (triangles, $\log y = 0.74 \log x + 0.46$, $r = 96$, $n = 0.92$, $p < 0.001$). Black, males; white, females. The dotted lines indicate the 95% confidence limits of the regressions.

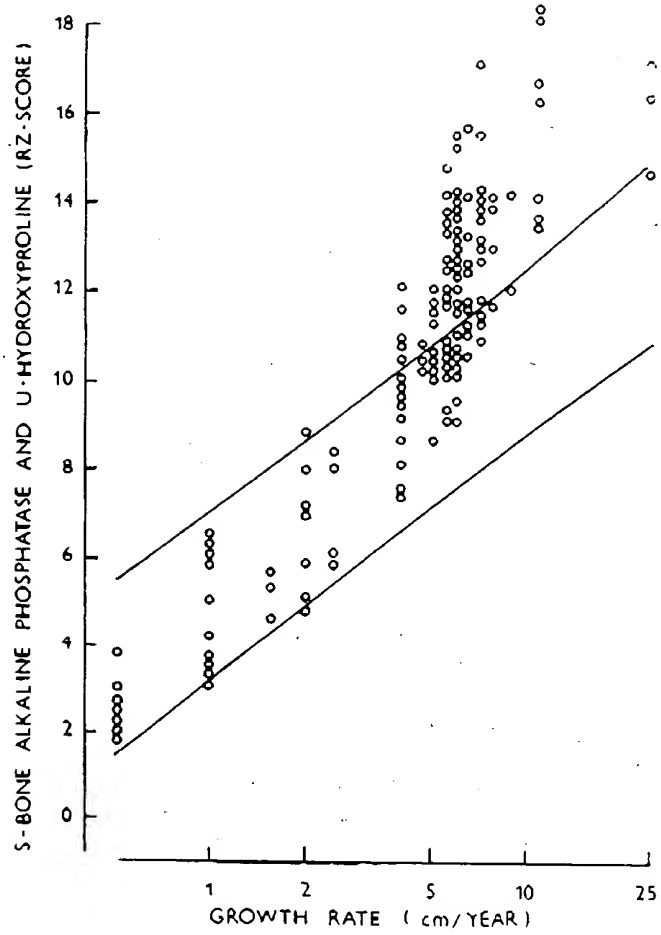

Fig. 6. Relationship between the mean growth rate for the particular age and sex, and the biochemical indices of bone remodelling. The lines indicate the 95% confidence limits of the regression of bone ALP on growth rate ($y = 5.00 \log x + 5.30$, $r = 0.91$, $n = 615$, $p < 0.001$). The circles indicate the individual values for urinary hydroxyproline excretion regressed on the growth rate ($y = 9.54 \log x + 4.45$, $r = 0.91$, $n = 142$, $p < 0.001$).

urinary hydroxyproline (y) in children ($\log y = 0.72 \log x + 1.25$, $r = 0.39$, $n = 49$, $p < 0.01$) was significantly different ($p < 0.001$) from that in the group from the end of puberty to 28 years of age ($\log y = 1.21 \log x + 0.48$, $r = 0.96$, $n = 50$, $p < 0.001$).

The biochemical indices of bone remodelling correlated significantly with the mean growth rate for the particular age and sex in children as well as following puberty (Fig. 6). Again, in children with high growth rate the urinary hydroxyproline excretion was more enhanced as compared with the bone ALP. This difference was not evident following the end of puberty. When the difference (HBP) was regressed on growth rate and puberty, the HBP remained a significant positive function of the growth rate and a significant negative function of puberty ($p < 0.001$).

Discussion

In this study, very constant bone ALP, TR-ACP and urinary hydroxyproline values are found between the ages of 29 and 45 in males as well as in females; male

and female values being very close to each other. Thus, this age group corresponds to the situation of a population class with minimum biological factors. It can therefore provide a reference population to which all other age groupings can be compared.

The aim of the present study was to compare the different biochemical indices of bone remodelling under physiological conditions. This is possible by use of the reference Z-score. The commonly used Z-score is the calculated deviation from the mean value of the distribution of normal samples expressed in standard deviation. In this study, the distribution of samples of the reference population is used (RZ-score). Originally, the concept of the upper reference limit was suggested and the 6th decade was chosen to provide a reference population in a study of the total serum ALP activity [13]. In that concept, however, the log-normal distribution of the values was not applied. The 6th decade is not suitable because of the sex-related differences for bone remodelling in this period.

The present results demonstrate that bone ALP, TR-ACP and urinary hydroxyproline values peak in the first two years of life and before the end of puberty. This is in good agreement with previous studies of bone ALP and urinary hydroxyproline in children and adolescents [5–9,13–16], as well as with anthropometric data [17]. Similarly, the additional bone ALP peak between 6 and 7 years of age precedes a temporary increase in total body calcium which is higher in boys than in girls [17]. In agreement with the morphometric data, a significant correlation is found between the growth rate and biochemical indices of bone remodelling in children and adolescents. The correlations of bone ALP with values for height and weight were less pronounced [16].

In this study, sex- and age dependency of TR-ACP is shown. Histochemically, TR-ACP is characteristically localised in the osteoclast. The number of osteoclasts rather than osteoresorption by osteoclasts seems to be indicated by the TR-ACP [2]. The enzyme activity is increased not only in children but in post-menopausal women as well. This corresponds to the changes in bone ALP and urinary hydroxyproline excretion, demonstrated in this study and by others [16,18], and with our data relating to an artificial postmenopause [19]. Individuals with any evidence of bone disease were not included in this study. However, greater than 30% of bone resorption is required before changes in bone density become visible by X-ray. This explains why women with increased biochemical indices of bone remodelling in the 6th decade were not excluded from this study.

The bone ALP, TR-ACP and urinary hydroxyproline values are closely correlated with each other. This is in good agreement with the concept of the coupling of bone formation to bone resorption [1]. However, in children the urinary hydroxyproline values are significantly higher when compared with those of bone ALP and TR-ACP. Sex hormones could be responsible for the observed evidence of differences in osteoclastic resorption efficacy in children and early postmenopause as compared with the reference population. Estrogens accelerate skeletal development and retard longitudinal bone growth [20,21] and reverse bone loss following the menopause. The sex hormones may have a direct inhibiting effect on bone resorption [22].

In both sexes, the highest urinary hydroxyproline excretion and peak rate of

growth occur almost simultaneously [9]. The urinary hydroxyproline peaks about three months before the menarche [23]. In this study, the dissociation between bone ALP and urinary hydroxyproline excretion peaks during the last year before the end of the mean pubertal age. At this age, the greatest dissociation between skeletal growth and mineralisation was reported [21]. This may in fact explain the peak incidence of forearm fractures observed in children of 10–14 years of age in both sexes [24].

The dissociation between urinary hydroxyproline and bone ALP in both sexes takes place also in the first year of age when the growth spurt is the highest. During this period, as in acromegaly [25], the 'additional' hydroxyproline may come to a greater extent from tissues other than bone since the connective tissues of the whole body are involved. It is unlikely that in pubertal and postmenopausal individuals the 'additional' hydroxyproline would originate from the non-dialyzable hydroxyproline from tissues other than bone. A further study of the hormonal influences on the biochemical indices of bone remodelling during puberty and following estrogen withdrawal is desirable.

The bone ALP was not related to the liver isoenzyme of ALP which shows a significant upward trend with age and appears to account for the increase in total ALP activity in older age groups [14].

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Appendix C

A Comparison of the Antiatherogenic Effects of Probucol and of a Structural Analogue of Probucol in Low Density Lipoprotein Receptor-deficient Rabbits

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Abstract

The efficacies of probucol and a close structural analogue as antioxidants in the prevention of atherogenesis in LDL receptor-deficient rabbits were compared. The antioxidant potency of the analogue in vitro was equal to that of probucol. Its biological availability was much greater: almost comparable concentrations in total plasma were achieved by feeding 1% probucol (wt/wt) and 0.05% analogue (wt/wt). Total plasma concentrations were comparable, but the concentration of probucol within the LDL fraction was about twice that of the analogue. Probucol slowed lesion progression by almost 50%, confirming earlier reports; the analogue, however, showed no detectable inhibitory effect on atherogenesis. Resistance of LDL to oxidation was measured at the end of the study by incubating it with Cu^{2+} and measuring the rate of diene conjugation. Probucol prolonged diene conjugation lag time from the control value of 130 min to values >1,000 min. The analogue approximately tripled the lag time (mean, 410 min) and yet failed to slow the atherogenic process. The results suggest that LDL resistance to oxidation must reach some threshold level before there is significant protection against atherogenesis. However, probucol has additional biological effects, possibly not shared by the analogue, that could contribute to its antiatherogenic potential. (*J. Clin. Invest.* 1994. 94:392–398.) Key words: atherosclerosis • macrophages • oxidation • antioxidant • drug therapy

Introduction

Many lines of evidence, based on studies in cell culture and in experimental animals, suggest that the conversion of native LDL to oxidatively modified LDL is a critical step in the atherogenic process (1, 2). Probably the most compelling evidence in favor of the oxidative modification hypothesis of atherosclerosis is that antioxidants have been shown in six of nine published trials (3–11) to slow the rate of progression of experimental atherosclerosis by more than 50% in LDL receptor-deficient rabbits (3–5) and in cholesterol-fed rabbits (7–9). One study of vitamin E in cholesterol-fed monkeys gave marginal results (11) and one rabbit study was negative (10). A majority of these studies made use of probucol as the antioxidant (3–6, 9, 10). Unfortunately probucol has a cholesterol-lowering effect and several additional biological effects that could very well contribute to or even be primarily responsible for the antiatherogenic effects observed. In some studies the cholesterol-lowering effect of probucol has been taken into account by treating the control group in such a way as to match cholesterol levels (3); in other studies this has not been done (4–6). The additional biological effects that may come into play include the ability of probucol to inhibit release of IL-1 (12), to increase the expression of cholesterol ester transfer protein (13, 14), and to act at an intracellular level to modify oxidative metabolism (15). The possibility that probucol's antiatherogenic effects are mainly related to its action as an antioxidant is strengthened by the fact that two other antioxidants have been shown to be effective against experimental atherosclerosis. Butylated hydroxytoluene (BHT)[1] was shown by Björkhem and co-workers to inhibit atherosclerosis in cholesterol-fed rabbits (7); *N,N'*-diphenylphenylenediamine (DPPD) was shown to be effective, again in cholesterol-fed rabbits, by Sparrow and co-workers (8). BHT is structurally closely related to probucol but it does not share the cholesterol-lowering effects of probucol. Thus, the Björkhem study is not confounded by the issue of cholesterol-lowering. On the other hand, BHT has not been tested to see whether it shares some of the other biological properties of probucol. Because of its structural similarity it may very well do so. DPPD is structurally quite different from BHT or probucol and again does not have any cholesterol-lowering effect. The fact that Sparrow and co-workers obtained a result rather similar to that obtained using probucol is consistent with the possibility that probucol works primarily as an antioxidant. However, it does not prove it beyond doubt. Thus there is a need to carry out studies with additional antioxidant compounds and to show unambiguously that the protective effects are due to the antioxidant effect. The present study explores the effects of a new antioxidant, a close structural analogue of probucol (Fig. 1).

Methods

Rabbits and diets. We studied 27 LDL receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits (12 females and 15 males)

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1. *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; DPPD, *N, N'*-diphenylphenylenediamine; TC, tyramine cellobiose; WHHL rabbit, Watanabe heritage hyperlipidemic rabbit.

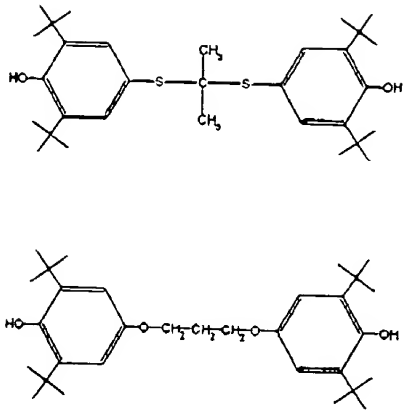

Figure 1. Chemical structures of probucol (*top*) and bis(3,5-diterbutyl-4-hydroxyphenylether)propane (BM15.0639) (*bottom*).

from four litters. The animals were divided into three groups, a control group (n = 10), a BM15.0639 group (n = 11), and a probucol group (n = 6). The groups were matched for litter, gender, and plasma cholesterol. Probucol (a gift from Merrell Dow Pharmaceuticals, Inc.) was added to the chow at a concentration of 1% (wt/wt). BM15.0639 [bis(3,5-di-tert-butyl-4-hydroxyphenylether)propane], a gift from Boehringer Mannheim, Mannheim, Germany is much more bioavailable than probucol, yielding similar plasma levels at 1/20th the dose of probucol. Therefore it was fed at a concentration of 0.05% (wt/wt). The drugs were added to the chow in diethyl ether and the control chow was similarly treated with plain solvent. The chow was dried for several days before use. The rabbits were fed the diets for 30 wk starting at 13 wk of age. The daily chow ration was gradually increased from 80 to 120 g as the rabbits grew. Extensive studies on the pharmacology and toxicology of BM15.0639 in rabbits were conducted by Boehringer Mannheim. No adverse effects at the doses used were found (H.A. Dresel, unpublished data), and none were found in the present studies.

Plasma lipids and determination of drug levels. Plasma samples were obtained every 2–4 wk after an overnight fast, and cholesterol levels were determined using an automated enzymatic technique (Boehringer Mannheim Diagnostics, Indianapolis, IN). The samples were used to determine drug levels in plasma. The concentrations of probucol and of BM15.0639 were determined using the same HPLC assay. In brief, plasma samples were extracted with methanol/acetone, 3:2 (vol/vol), with addition of 2-pentanone-bis(3,5-di-*t*-butyl-4-hydroxyphenyl)mercaptole as internal standard, and partitioned into heptane. The samples were analyzed by HPLC on a C_{18}-reversed phase column eluted with acetonitrile/heptane/0.1 M ammonium acetate, 92:6:2 (vol/vol). Absorption at 254 nm for probucol and at 234 nm for BM15.0639 was measured. The determination of drug levels in LDL was done using the same method. The concentration of probucol and BM15.0639 in tissue was determined by HPLC after enzymatically digesting, homogenizing, and extracting the tissue (16). In brief, 100 mg of tissue was incubated with 1 ml bacterial collagenase (type I, 5 mg/ml) and 0.5 ml porcine lipase (2 mg/ml) for 3 h at 37°C. Enzymes were from Worthington Biochemicals (Freehold, NJ). To each sample was added 50 μl of ascorbic acid (10 mg/ml). After this digestion the samples were homogenized using a ground-glass pestle-tube system. Internal standard was added and the sample was extracted twice with hexane. The combined hexane phases were dried under a stream of nitrogen. The extracted material was resolubilized in HPLC mobile phase and analyzed as described for plasma drug levels.

Isolation of LDL and LDL modification. LDL (d = 1.021–1.060 g/ml) was isolated by sequential ultracentrifugation from plasma collected into EDTA after an overnight fast (17). Protein was determined by the method of Lowry et al. (18) with BSA as a standard. At the end of the isolation LDL was extensively dialyzed against PBS containing 2 mM EDTA. LDL was subjected to prooxidative conditions to study its resistance to oxidation as follows: preceding the use of LDL, EDTA was removed by dialysis against PBS. Unlabeled or ^{125}I-labeled LDL was diluted in Ham's F-10 medium (100 μg LDL protein/ml) and then was incubated in 60-mm plastic dishes with confluent rabbit aortic endothelial cells for 18 h or incubated in PBS in the presence of 5–10 μM $CuSO_4$. The formation of conjugated dienes was measured as the increase in absorption at 234 nm. Lag times were determined graphically as the timepoint at which the tangent to the curve during the maximum slope of the propagation phase intercepted the time axis. Absorption at the beginning of the reaction was set to zero. Thiobarbituric acid–reactive substances were measured as an index of the degree of lipid peroxidation (19). The extent of LDL oxidation was also assessed in terms of the increase in its rate of degradation by macrophages: 10 μg of ^{125}I-LDL (native or modified) in 0.5 ml DME was added to mouse peritoneal macrophages in 24-well dishes and incubated at 37°C for 5 h. Trichloroacetic acid–soluble radioactivity in the medium and cell-associated radioactivity were then determined.

Extent of aortic lesions. Each rabbit was given 1,000 IU heparin and then was deeply anesthetized with sodium pentobarbital (50 mg/kg). The systemic circulation was perfused with 2 liters of isotonic PBS containing 2 mM EDTA through a large-bore cannula introduced into the apex of the left ventricle while collecting the effluent from the severed right ventricle. The aorta was then fixed in situ with half-strength Karnowsky's solution for 20–25 min. A perfusion pressure of 80 mm Hg was maintained during the entire procedure, using large elevated reservoirs. The entire aorta was removed and cleaned of loose adventitial tissue. The thoracic and abdominal aortas were divided 5 mm proximal to the celiac artery. Each segment was opened longitudinally and fixed in half-strength Karnowsky's for an additional 24 h. The aortas were stained with Sudan IV, pinned flat on wax beds, and covered with PBS. They were then photographed and digitally recorded using a Cohu solid-state camera connected to a personal computer via a data-technology 2851 frame grabber board. The captured image was processed using image pro II software (Media Cybernetics, Inc., Silver Spring, MD). The areas of sudanophilic lesions and the total area of each aortic segment were determined (20). The extent of lesions was expressed as percent of total aortic surface area involved.

Metabolic studies. In subsets of four rabbits from each of the three different treatment groups, arterial LDL degradation rates were determined at killing. LDL was isolated from pools of plasma of each group as described above. The LDL was first iodinated conventionally with ^{125}I using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodogen; Pierce Chemical Co, Rockford, IL). It was then covalently linked to ^{131}I-tyramine cellobiose (^{131}I-TC), a lysosomally trapped tracer, with cyanuric chloride as described (21). After dialysis against PBS containing 2 mM EDTA, <1% of the radioactivity of each isotope was soluble in 10% (wt/vol) trichloroacetic acid. Radioactivity extractable into chloroform/methanol, 1:1 (vol/vol), was 2.41±0.34% for ^{125}I and 1.47±0.33% for ^{131}I. The specific activities of ^{125}I and ^{131}I-TC ranged from 281 to 462 and 65.4 to 126 cpm/ng protein, respectively. The LDL was used 2–3 d after labeling, which was 3–4 d after initial isolation. The doubly labeled LDL ($6.97\pm1.29 \times 10^6$ cpm of ^{125}I and $1.80\pm0.42 \times 10^6$ cpm of ^{131}I) was injected intravenously after the rabbits had been injected with 3 mg of NaI to prevent uptake of radioiodide by the thyroid. The animals received homologous LDL. The plasma decay of labeled LDL was followed over 72 h by obtaining 12 serial samples of blood beginning at 10 min after injection. At killing the systemic circulation was perfused with PBS; the aortas were fixed in situ, dissected, stained, and photographed; and the sudanophilic lesion area was determined as described above. The aortic arch was separated from the descending thoracic aorta 1–2 mm below the ductus scar. Sudan positive atherosclerotic lesions and negative nonlesioned areas were cut out of each aortic segment and weighed. The ^{125}I and ^{131}I contents of tissue and plasma samples were measured in a well-type γ-scintillation-counter (Compu Gamma; LKB Instruments Inc., Gaithersburg, MD) with cor-

Table I. Inhibition of Cu^{2+}- and Endothelial Cell–promoted LDL Oxidation In Vitro

	Additions	TBARS	Degradation by macrophages
		nmol MD/Aml	*μg/5hr=mg protein*
Native LDL	None	1.4	0.8
LDL incubated with endothelial cells		40	8.85
	1 μM probucol	42	9.15
	2.5μM probucol	37	7.30
	5 μM probucol	6	1.20
	None	39	9.00
	1 μM BM15.0639	Nondetectable	1.05
	2.5 μM BM15.0639	Nondetectable	1.45
	5 μM BM15.0639	Nondetectable	1.13
	None	48	6.75
LDL incubated with Cu^{2+}			
	1 μM probucol	52	6.05
	2.5 μM probucol	52	4.65
	5 μM probucol	23	0.86
	None	50	6.45
	1 μM BM15.0639	55	3.95
	2.5 μM BM15.0639	41	0.95
	5 μM BM15.0639	6	1.00

^{125}I-LDL (100 μg/ml) was incubated for 18 h with either 5 μM Cu^{2+} or endothelial cells in F-10 medium. The extent of LDL oxidation in the presence of probucol or BM15.0639 was then assessed by measuring thiobarbituric acid–reactive substances (TBARS) and the rate of degradation by macrophages.

rections for overlap of the energy spectra of the two isotopes, for background activity, and for isotopic decay. The rate of LDL catabolism in the whole body and in aortic tissues was then determined as described in detail previously (22). The procedure is based on the nearly complete lysosomal retention of TC after TC-LDL degradation, i.e., TC acts as a "trapped ligand." The arterial content of conventionally iodinated ^{125}I-labeled LDL, i.e. undegraded native LDL, was used to correct for ^{131}I-TC covalently bound to undegraded LDL within the artery, thus allowing assessment of the fraction of LDL degraded. ^{125}I derived from degradation products of conventionally labeled LDL are not trapped intracellularly and are not retained in tissue fixed with half-strength Karnowsky's (22, 23).

Histology. For immunohistochemistry, serial 5-μm thick sections were cut from the paraffin-embedded aortae and stained with RAM-11 (24), a monoclonal antibody specific for rabbit macrophages; or HHF-35 (25), a monoclonal antibody against actin; or MDA2 (26) a mouse monoclonal antibody against MDA-LDL as described in detail previously (27). We used an avidin-biotin-alkaline phosphatase system (Vector Laboratories, Inc., Burlingame, CA) for visualization. To investigate lipid distribution in tissue, 10-μm thick frozen sections were cut from OCT-embedded aortae. Lipids were stained with 0.4% Sudan black B in propylene glycol (28).

Statistical analysis. All results are expressed as mean±SEM. Differences between treatment groups are assessed by analysis of variance and covariance. The statistical analyses were performed using BMDP statistical software (29).

Results

Inhibition of LDL oxidation in vitro. The potencies of BM15.0639 and probucol in the inhibition of LDL oxidation in vitro were compared. As shown in Table I, probucol showed a partial inhibition of LDL oxidation by endothelial cells at 2.5 μM and almost completely blocked oxidation at 5 μM. The

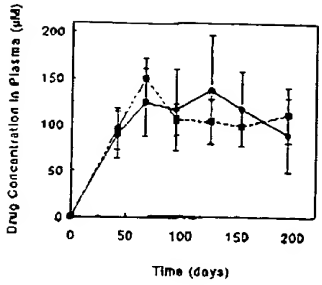

Figure 2. Plasma concentrations of BM15.0639 and of probucol in WHHL rabbits as a function of days of treatment. Plasma samples of animals were taken at different timepoints and analyzed for drug content by HPLC (see Methods). The data shown are mean plasma concentrations ±SEM. ——●——, BM15.0639 (n = 11); ---■---, probucol (n = 6).

analogue was even more potent in this in vitro test system, inhibiting oxidation of LDL almost completely even at 1 μM.

Oxidation induced by incubation with copper ions was also inhibited effectively by both compounds. Probucol again showed inhibition at 2.5 μM and almost completely blocked at 5 μM; the analogue showed a definite effect a 1 μM and was completely inhibitory at 2.5 μM. It should be noted that the concentrations of drug actually reached within the LDL particles under these conditions is not known; the drugs were simply added in ethanolic solution but no measurements of the fraction entering the LDL particles were made.

Both drugs were also shown to inhibit LDL oxidation assessed in terms of increased electrophoretic mobility and the ability of the oxidatively modified LDL to induce accumulation of cholesterol esters in macrophages. These results were consonant with those described above, i.e., the analogue had a somewhat greater potency than that of probucol itself (data not shown).

Plasma drug levels and degree of protection of plasma LDL against oxidative modification ex vivo. Previous studies of pharmacodynamics at Boehringer Mannheim laboratories had shown that BM15.0639 was much more readily absorbed than probucol (H. Dresel, personal communication). In fact, comparable blood levels were reached at 1/20th the dose of probucol. As shown in Fig. 2, total plasma concentrations of the analogue and of probucol were almost exactly the same when probucol was included in the diet at 1% (w/w) and the analogue at 0.05% (w/w). However, even though the mean total plasma concentrations of the two drugs were similar, the concentration of probucol in the LDL particles was approximately twice as high as that of the analogue: 27.8 nmol probucol/mg LDL protein versus 12 nmol of analogue/mg LDL at 30 wk. Both drugs were transported almost exclusively in lipoproteins, i.e., almost none was found in the 1.21 bottom fraction. The higher concentrations of probucol per LDL particle reflected in part the decrease in total plasma lipoproteins in the probucol-treated group (i.e., the drug was distributed among a smaller total number of lipoprotein particles) and the fact that a higher percentage of the total plasma lipids was found in the LDL fraction in the probucol-treated groups (i.e., the VLDL dropped to a greater extent than did the LDL so that the fraction of total plasma lipids represented by the LDL increased as did its share of the lipophilic drug).

LDL was isolated from the plasma at the end of the feeding period and tested for its resistance to oxidation ex vivo. The

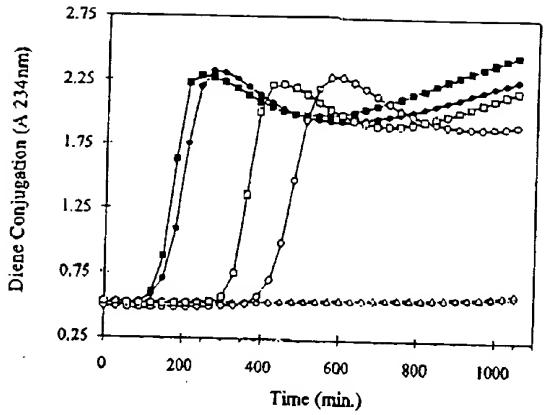

Figure 3. Real-time rate of Cu^{2+}-catalyzed diene conjugation in plasma LDL isolated from control rabbits and rabbits treated with BM15.0639 or probucol. LDL isolated from rabbits after 30 wk of treatment was pooled and subjected to Cu^{2+}-promoted oxidation. The formation of conjugated dienes was measured by measuring the absorption at 234 nm. Control LDL + 10 μM Cu^{2+} (——■——). control LDL + 5 μM Cu^{2+} (——●——). BM15.0639 + 10 μM Cu^{2+} (——□——), BM15.0639 + 5 μM Cu^{2+} (——○——), probucol + 10 μM Cu^{2+} (---△---), probucol + 5 μM Cu^{2+} (---○---).

samples were incubated in the presence of 5 or 10 μM $CuSO_4$ and the rate of conjugated diene formation was followed by measuring absorption at 234 nm. As shown in Fig. 3, LDL from animals treated with probucol was almost completely protected against oxidative modification for over 1,000 min under these conditions. In contrast, LDL from the animals treated with the analogue, although showing a definite extension of lag time compared with control LDL, was much less well protected. The diene conjugation lag time for the analogue-treated animals was 313 min in the presence of 10 μM copper and 410 min in the presence of 5 μM copper. The control samples showed lag times of only a little over 100 min.

To determine whether the results obtained at the termination of the study were representative or not, we studied a small number of animals fed the drugs for 14 d or for 9 wk. As shown in Table II, the results were very similar to those obtained at the end of the study, i.e., the lag time for diene conjugation was prolonged to a much greater extent in the LDL from probucol-treated animals than in the LDL from the analogue-treated animals and both were considerably longer than the value in the controls.

Effects of treatment. None of the animals showed any signs of toxicity and the weight gain in all groups was similar. Total plasma cholesterol in the untreated controls was 788±113 mg/dl during the studies. The analogue did not decrease plasma cholesterol at all (745±102 mg/dl) but probucol, as expected, did (581±115 mg/dl during the study). Lipoprotein profiles, carried out at time zero and after 18 wk of feeding showed that HDL accounted for only < 2% of the total plasma cholesterol and there were no significant differences in HDL levels between the probucol-treated and the analogue-treated groups; both showed a drift downward during the course of the study. The LDL fraction accounted for 27% of the total plasma cholesterol

Table II. Effects of Probucol and of BM15.0639 on Cu^{2+}-catalyzed Oxidation of Plasma LDL after 2, 9, and 30 wk of Treatment

Length of treatment	Lag times		
	Control	BM15.0639	Probucol
	min	*min*	*min*
14 d	137.1 (2)	403.7 (3)	935.8 (3)
9 wk	135.0 (2)	496.7 (3)	928.3 (3)
30 wk	123.0 (10)	410.0 (11)	> 1080 (6)

LDL (100 μg/ml) was incubated in PBS and oxidation was initiated by adding 5 μM Cu^{2+}. Lag times were determined as described in Methods. Values in parentheses are number of animals.

at the beginning of the study. However, because there was a large decrease in VLDL cholesterol in the probucol group (~ 50%), LDL accounted for a larger fraction of total plasma cholesterol at the end of the study. As discussed below, these shifts are relevant to the observed changes in drug concentration within lipoprotein particles.

The extent of sudanophilic lesions in the aortas of the three groups is shown in Table III. The quantification of surface lesion areas by digital imaging showed significantly less atherosclerosis in the probucol-treated animals compared with the control animals ($P < 0.01$). The total arterial surface area involved in lesions was reduced by almost 50%. The reduction of arteriosclerosis was highly significant ($P < 0.01$) in all three segments of the aorta. The slowing of the atherosclerotic process was most pronounced in the descending thoracic aorta (−71%). On the contrary, in rabbits treated with BM15.0639 the extent of aortic lesions was unchanged compared with the control group. The small differences in individual segments of the aorta were statistically not significant.

In an attempt to assess the extent to which the decrease in plasma cholesterol caused by probucol might have contributed to its observed antiatherogenic effect, a statistical analysis of variance with cholesterol as covariant was performed. Because there was no difference in lesion area between the control group and the analogue-treated group, the results in those two groups were pooled (32.8±6.9% of aortic surface covered by lesions).

Table III. Extent of Aortic Lesions in WHHL Rabbits after Treatment with BM15.0639 or Probucol

Experimental group	Extent of aortic lesions			
	Total aorta	Arch	Descending thoracic	Abdominal
	% of surface area involved			
Untreated (n = 10)	32.8±5.8	66.0±11.6	29.1±9.0	19.2±4.1
BM15.0639 (n = 11)	32.8±4.9	71.5±13.8	27.0±3.8	17.9±3.9
Probucol (n = 6)	17.8±3.8	47.6±10.4	8.4±9.4	10.9±4.7

Surface areas of sudanophilic lesions were determined by digital imaging and expressed as percentage of total arterial surface. Probucol treatment decreased the extent of arteriosclerotic lesions significantly ($P < 0.01$) compared with untreated and BM15.0639-treated animals.

Table IV. Rates of LDL Degradation in the Aorta (fraction of plasma LDL pool degraded × 10^5 per g of tissue per day)

	Arch	Descending thoracic	Abdominal
Untreated (n = 4)	24.8±3.6	21.0±11.9	10.9±1.9
BM15.0639 (n = 4)	18.4±5.4	21.5±12.6	8.0±4.7
Probucol (n = 4)	13.1±4.5	10.0±8.9	5.9±2.3

Analysis of variance with repeated measures was used to compare data between groups. Differences between probucol-treated and untreated animals were significant (P = 0.001). BM15.0639-treated animals were not different from untreated at P = 0.372.

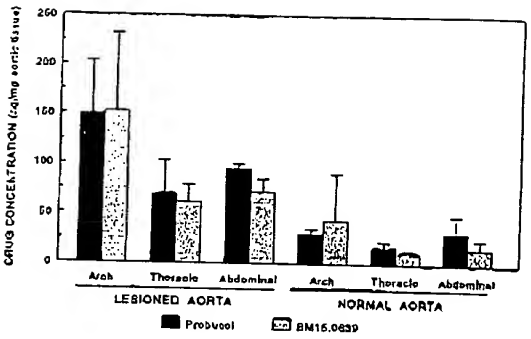

Figure 4. Concentration of probucol and BM15.0639 in aortic tissue. LDL receptor-deficient rabbits were fed a diet containing 1% probucol or 0.05% BM15.0639 for 18 wk. Aortas were removed and divided into arch, descending thoracic, and abdominal aorta. Drug concentrations in lesioned and unlesioned tissue were determined separately as described in Methods. The results shown are means of three animals per group.

Before adjustment for plasma cholesterol levels, lesion area in the probucol-treated group was 17.8±3.8% and after adjustment 19.2%. The latter was still significantly different from the value in the control group (32.4% after adjustment) at the $P < 0.01$ level.

Metabolism of LDL in the whole body and within the arterial wall was measured in three subgroups of four WHHL rabbits each. The rate of LDL catabolism in the whole body showed similar fractional catabolic rates (control rabbits, 0.016±0.001 h^{-1}; BM15.0639-treated rabbits, 0.015±0.001 h^{-1}; probucol-treated rabbits, 0.012±0.002 h^{-1}) The rate of LDL degradation within lesions, on the contrary, was significantly decreased in animals treated with probucol (Table IV). Animals treated with BM15.0639 showed no significant difference in fractional catabolic rates within lesions.

Drug levels in aortic tissue and histology. The concentrations of probucol and BM15.0639 in aortic tissue were determined in a separate set of animals after 2 and 18 wk of treatment. At both time intervals, the concentrations of the compounds were almost identical, both in normal aortic tissue and in lesioned areas. After 18 wk of treatment both compounds were found at much higher concentrations within lesions than in normal aorta. The drug levels measured after 18 wk are shown in Fig. 4.

The cellular composition of lesioned arterial tissue was examined by immunohistology. We used the monoclonal antibodies RAM-11, HHF-35, and MDA2, specific for macrophages, smooth muscle cells, and malondialdehyde-modified LDL, respectively. Comparing lesions at similar stages of development, no obvious treatment effect on cellular composition was seen. To examine the distribution of lipids within the arterial wall, frozen sections were prepared and stained with Sudan black B. A representative number of sections from all three segments of the aorta were analyzed. No obvious change in lipid distribution was found among the three treatment groups.

Discussion

These results confirm the effectiveness of probucol as an antiatherogenic drug in LDL receptor-deficient rabbits. As in previous studies (3–5), the probucol-treated animals showed about a 50% inhibition in the extent of lesions, and the LDL from these animals strongly resisted oxidative modification in vitro. As in the studies of Carew et al. (3), we were able to show that the rate of uptake of injected native LDL into atherosclerotic lesions was sharply reduced in the probucol-treated animals. This is very likely because probucol inhibits oxidation of LDL, for the following reasons. First, earlier studies have shown that most of the uptake of injected native LDL into lesions in these rabbits is attributable to uptake into macrophages (30). Second, Watanabe rabbits express almost no functional native LDL receptors and, in any case, these would be expected to be downregulated in the face of the very high plasma LDL concentrations found. In fact, in situ hybridization studies (31) have shown that the LDL receptor *is* markedly downregulated in atherosclerotic lesions but that the acetyl LDL receptor is strongly expressed. Thus it seems reasonable to conclude that most of the uptake of injected native LDL into lesions in this and related studies is attributable to macrophage uptake via the acetyl LDL receptor, which means the LDL must have been first converted from the native form to the oxidatively modified form. Probucol would be expected to inhibit this conversion and the decreased incorporation into lesions in the probucol-treated animals is consistent with this interpretation. Alternative interpretations, however, are possible as discussed below.

The probucol analogue studied here (BM15.0639) was just as effective as an antioxidant in vitro as probucol. Because of the much greater bioavailability of the analogue and because we wanted to try to achieve comparable plasma levels of the two compounds, the analogue was fed at only 0.05% in the diet whereas probucol was fed at 1% (wt/wt). The *total* plasma concentrations of the two drugs at the end of the studies were in fact about the same. Lesion development, however, was not affected by the treatment with BM15.0639. The rates of LDL degradation within arterial lesion sites of BM15.0639-treated rabbits was also not significantly different from control animals, suggesting that oxidation of LDL was not being inhibited. LDL isolated from plasma of the analogue-treated animals at the end of the study was protected against oxidation, but not nearly as well as LDL from probucol-treated rabbits. Whereas probucol prolonged the diene conjugation lag time to values of ≥1,000 min, the analogue only extended the lag time to ~ 400 min (compared with the normal value of 130 min). Although the *total* plasma concentrations were similar, the number of molecules of probucol per LDL particle was twice the number of analogue molecules per particle. This was accounted for by two

factors: (*a*) the total plasma cholesterol of the probucol-treated animals fell by 25% so that the drug was distributed into a smaller total number of lipoprotein particles than in the case of the analogue-treated group and (*b*) the fraction of the total plasma cholesterol present in the LDL fraction increased by almost 50%. The latter was mainly due to a large drop in VLDL cholesterol. Thus, the larger number of probucol molecules in the LDL may be enough to explain the difference in degree of protection against oxidation. It is also possible that metabolites of probucol carried in LDL (not measured in these studies) may make a contribution to the protection against oxidation seen in the probucol-treated group. The analogue probably is not metabolized in the same way as probucol. Lacking the sulfur atoms in the bridge (see Fig. 1), its metabolism may be limited to the aromatic rings. Whereas metabolites of probucol were detected by HPLC, no metabolites of BM15.0639 were noted. Whatever the mechanism involved, the key question is whether the difference in degree of protection against oxidative modification ex vivo is sufficient to explain the ineffectiveness of the analogue in slowing the progression of atherosclerotic lesions. Compatible with this conclusion is the fact that the incorporation of injected native LDL into arterial lesions was *not* inhibited in the analogue-treated rabbits.

Little or no information is available that relates effectiveness of antioxidants in vitro to their antiatherogenic potential. We do not know whether there is a graded relationship or a threshold relationship. The latter possibility is a real one. Consider the transport of LDL into and out of the arterial wall. LDL particles will on the average reside within the artery wall for some defined time interval (mean residence time). Schwenke and Carew (32) have estimated the mean residence time of LDL particles in the normal rabbit aorta and in the aorta of animals with experimental atherosclerosis. Under normal conditions, with a short residence time within the artery wall, LDL might undergo very little oxidative damage during transit. Once it reenters the plasma compartment, the chances that it will reenter the artery are extremely small, since only a very small fraction of LDL turnover is attributable to arterial uptake. If the degree of oxidation of a particular LDL particle goes beyond a certain point during its passage through the artery wall, that LDL may become a target for uptake via scavenger receptors or it may undergo complexing with connective tissue matrix, or it may aggregate with other LDL particles. In that case, its fate may be to stay indefinitely within the artery wall and wind up being taken up by macrophage scavenger receptors. The central point here is that in order to be effective, an antioxidant residing in the LDL particle may need to protect it for a length of time near to or greater than the residence time of LDL within the artery wall. That time may not be the same as the diene conjugation lag time in absolute terms, because the latter is measured under artificial in vitro conditions that may or may not reflect the pro-oxidant "stress" within the artery wall, but there might be some proportionality. It is conceivable that there is a threshold level of protection that would represent the minimum necessary to exert an antiatherogenic effect. In this connection, a recently completed study of the effectiveness of probucol in *Macaca nemestrina* may be pertinent. Sasaharu and co-workers (33) showed that probucol (1% wt/wt in the diet) inhibited lesion formation by ~ 50% in the thoracic aorta ($P < 0.001$). No effects were seen in the abdominal aorta nor in the iliac arteries, possibly because lesions were more advanced in these arterial segments. The investigators measured diene conjugation lag time in the LDL isolated from these animals at the end of the study. What they found was that there was a negative correlation between the extent of lesion area and the prolongation of the diene conjugation lag time. They suggested that diene conjugation lag times needed to be ≥ 400 min in order to significantly inhibit lesion formation. This value is near the diene conjugation lag time achieved with the analogue in the present studies, an effect that was not associated with inhibition of lesion formation. This should not be overinterpreted because of the species difference but the coincidental finding of a similar "threshold" in prolongation and lag time is worth noting.

Although the proposed antioxidant mechanism is appealing, it is still unclear whether this is the major underlying mechanism of probucol's action. This is especially true, because BM15.0639 failed to slow the progression of atherosclerosis despite protecting LDL against oxidation ex vivo. The degree to which LDL was protected was remarkably high compared with maximally achievable protection with natural antioxidants, such as vitamin E. Probucol has a number of additional effects which therefore need to be considered. First, probucol does have a cholesterol-lowering effect and cholesterol levels were somewhat lower in the probucol-treated group. However, the difference was small and, as discussed above, correction for the effect of the degree of hypercholesterolemia on the extent of lesion formation accounts for only a very small part of the probucol effect in lesion formation. In the studies of Carew et al. (3) the control rabbits were treated with a small dose of lovastatin, just enough to match the plasma cholesterol levels in those treated with probucol. Thus, a difference in cholesterol levels did not contribute to the antiatherogenic effect of probucol in those studies. In the studies of Sparrow et al. (8), using DPPD, and the studies by Björkhem et al. (7), testing BHT, there were no differences in cholesterol level and the effectiveness of those two antioxidants in cholesterol-fed rabbits is clearly not due to a cholesterol-lowering effect. For all of these reasons it does not seem likely that the different results in the present studies are due to the small decrease in cholesterol levels induced by probucol.

A second category of explanation for the difference in effectiveness of the two compounds against atherogenesis is that probucol has additional biological properties that may not shared by the analogue. As pointed out in the introduction, several of these properties could be relevant to its antiatherogenic effect (12–15). The analogue has not yet been tested to determine whether it shares these properties with probucol.

Whatever the explanation of the unexpected ineffectiveness of BM15.0639 against atherogenesis, these negative results are instructive. Many investigators, including ourselves, have tended to assume that the relative effectiveness of antioxidants as antiatherogenic agents would parallel their effectiveness in protecting LDL in ex vivo measurements of oxidation under controlled conditions. Clearly things are more complex. Much more work is needed to sort out what the true relationship is and to search for other relevant variables.

Acknowledgments

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Appendix D

Jikken Igaku (Experimental Medicine), Vol. 6, No. 14, 1988, page 130 (1446), left col., lines 9-11

I have seen, in addition to the two cases reported by Matsuzawa et al, five cases of hyper-high-density-lipoproteinemia with turbidity of the cornea. Of the five cases, two were accompanied by severe coronary disease.

No. 7

おわりに

動脈硬化発生機構の解明において重要な位置を占める泡沫細胞の由来は，少なくとも初期段階には主として，単球から分化したマクロファージであることは周知の事実となってきた．またその泡沫化機構については，変性 LDL の研究をはじめとして徐々に解明されつつあり，今なお多数の研究者のテーマとして研究が続けられている．今後 *in vitro* のレベルでの研究がよりいっそう進展するのとともに，*in vivo* のレベルへと展開していくことが期待される．

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〈著者プロフィール〉
昭和59年山口大学医学部卒業．その後京都大学医学部内科勤務を経て，63年から京都大学医学部内科第3講座にて研究活動を始める．現在，変性 LDL 特にタバコ煙抽出物修飾 LDL によるマクロファージの泡沫化機構について研究中．今後，生体内での変性 LDL の証明に努めるとともに，動脈硬化の発生機構に続く進展機構の解明へと進めて行けるよう努力したいと思っている．

Appendix D.

リポ蛋白質の代謝と異常—LDL と HDL

船橋 徹　松沢佑次

血液中の脂質（コレステロール，トリグリセライド）はリポ蛋白質として輸送されている．体内で合成された脂質の輸送系路は大きく2つに分けられ，1つは肝で合成された VLDL (very low density lipoprotein) から LDL (low density lipoprotein) を経て末梢の LDL レセプターに取り込まれる系であり，もう1つは HDL の代謝系路である．HDL の代謝過程は未だ十分解明されていないが，末梢から肝への reverse cholesterol transport の役割が提唱されている．臨床的に LDL，HDL の代謝異常により動脈硬化疾患が生じることはよく知られている．遺伝的にこれらのリポ蛋白質代謝に関与するアポ蛋白，酵素などの異常症が存在し，そのいくつかは分子遺伝学的異常が確認されているので紹介する．

はじめに

血液中の脂質は，両親媒性の蛋白質（アポ蛋白質）との複合体であるリポ蛋白質として存在している．リポ蛋白質は中心部にコレステロール・エステルとトリグリセライドを含み，表面を遊離コレステロールとリン脂質からなる単分子膜でおおわれた球状粒子で，アポ蛋白質は膜表面に結合してリポ蛋白質の安定化やリポ蛋白質代謝上の種々の機能を果たす．肝で合成された脂質は超比重リポ蛋白質 (very low density lipoprotein : VLDL) として分泌される．VLDL 中のトリグリセライドは毛細血管壁に存在するリポ蛋白質リパーゼ (lipoprotein lipase : LDL) によって水解され，VLDL は次第にその径を小さくしコレステロール・エステルに富む低比重リポ蛋白質 (low density lipoprotein : LDL) となる．VLDL にはアポ A I，C II，C III，E，B (B100) などが結合しているが，分子量の大きい (514kD) アポ B 以外は VLDL の水解過程でリポ蛋白粒子より解離する．アポ B は LDL 1粒子あたり1モル存在し，細胞表面に存在する LDL レセプターのリガンドとなる．LDL レセプターは肝，腎，副腎，精巣，脳など多くの臓器で発現しているが，肝における発現量が最も多く（単位重量あたりでは副腎が多い），血液中からの LDL の除去に大きな役割を果たしている．LDL レセプターの発現量は細胞内コレステロール量により調節を受ける．血液中の LDL 量は主としてアポ B の合成量と LDL レセプター量により規定され，LDL の血中でのうっ滞は動脈硬化症を促進すると考えられている．

HDL はおもに肝，小腸で合成されると考えられているが，その主要アポ蛋白質のアポ A I，A II も肝と小腸で合成される．ラットの肝灌流実験により HDL は遊離コレステロールとリン脂質膜からなる円盤状の粒子 (nasent HDL) として分泌されると考えられているが，HDL 表面には lecithin-cholesterol acyltransferase (LACT) が存在し，遊離コレステロールをエステル化し，それを粒子中心部に取り込むことによって HDL は球状の成熟粒子となる．また HDL の一部は，VLDL などの水解過程で余剰の膜成分からも形成されると考えられている．HDL は臨床研究から動脈硬化を防御するリポ蛋白と推察されてきたが，HDL の生理的機能は必ずしも明らかにされていない．

コレステロールの異化を触媒する酵素は肝にのみ存在し，末梢細胞内に存在する余剰のコレステロールは

〔キーワード〕
LDL : low density lipoprotein
HDL : high density lipoprotein

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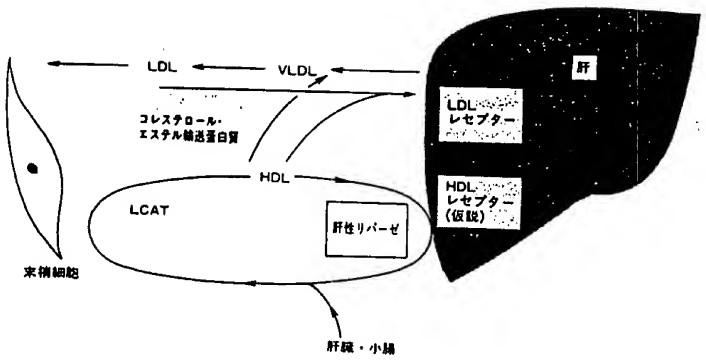

図1　LDL，HDL の代謝

HDL を介して肝に運ばれるということが種々の成績により少しずつ明らかとなっており，これが動脈硬化の防御とつながるのではないかと考えられている．血液中には neutral lipid を転送する蛋白質（コレステロール・エステル転送蛋白質(cholesterol ester transfer protein：CETP)）[1]が存在し，HDL 中のコレステロール・エステルを VLDL，LDL に転移し，トリグリセライドを HDL の方向に転移する反応を触媒する．従って HDL 中のコレステロール・エステルの少なくとも一部は LDL → LDL レセプターを介し，肝に輸送される．また肝細胞には HDL を結合する蛋白（HDL レセプター）が存在し，直接 HDL から肝細胞にコレステロール・エステルが転送されるという経路も存在する可能性がある[2]．末梢細胞から HDL を介し肝細胞へとコレステロール・エステルを輸送する逆転送系(reverse cholesterol transport) が動脈硬化などの脂質蓄積状態の防御機構として注目されてきたのである．

以上 LDL，HDL の役割および生体内の動態を述べたが（図1），多くの疫学的研究により高 LDL 血症，低 HDL 血症は動脈硬化症，特に冠動脈疾患のリスクファクターと考えられている．また LDL，HDL の遺伝的代謝異常により，冠動脈疾患や角膜混濁，黄色腫などの脂質蓄積症状をきたす疾患が知られている．これらのいくつかはその遺伝的本態が明らかにされているので紹介する．

1．HDL 代謝異常

1）HDL が低下する病態（表1，2）

i）LCAT 欠損症

LCAT 欠損症は1967年 Norum ら[3]によって発見された角膜混濁，貧血，腎障害を主症状とする常染色体劣性遺伝疾患であり，現在までに12家系26例の報告がある．LCAT は分子量63,000の糖蛋白質で，レシチンの2位の脂肪酸を遊離コレステロールに移し，コレステロール・エステルを形成する反応を触媒する酵素である．この酵素反応は HDL の主要アポ蛋白であるアポ AI により活性化される．LCAT は肝で合成され，疎水性に富みリポ蛋白との親和性が高い．178～183残基のアミノ酸配列は中心に Ser を含みウシ膵リパーゼの活性中心と相同であり，そのC端およびN端側には疎水性のアミノ酸配列が位置し，リポ蛋白と結合すると考えられる[4]．血液中のリポ蛋白質中のコレステロール・エステルのかなりの部分が LCAT によって生成されると考えられている．LCAT 欠損患者の血清中では遊離コレステロールとレシチンが増加し，コレステロール・エステルは減少している．総コレステロールに対するコレステロール・エステル比は正常では70～75% であるのに対し，患者では0～30% に減少している．このエステル化率の減少は HDL 分画で特に著しく，HDL コレステロールは著減している．HDL 分画にはアポ E を含む円盤状粒子が存在し，これは LCAT 阻害下に肝灌流液に出現する nasent

表1　HDL 欠損～低下症の臨床所見

	角膜混濁	黄色腫	腎糸球異常 神経障害	肝脾腫	早発性 冠動脈 硬化症
家族性 LCAT 欠損症	+	−	−	±	−
家族性 ApoAI-CIII 欠損症 (Schaefer)	+	−	−	−	+
家族性 ApoAI-CIII 欠損症 (Norum)	+	+	−	−	+
Apo AI $_{Milano}$*	−	−	−	−	−
Tangier 病	+	−	+	+	+
HDL deficiency with planar xanthomas	+	+	−	+	+
Familial hypoalpha-lipoproteinemia*	−	−	−	−	+
Fish-eye disease	+	−	−	−	−

＊　ヘテロ接合体

表2　HDL 欠損～低下症の血清脂質・アポ蛋白値

	Chol	TG	HDL Chol	ApoA I	ApoA II
家族性 LCAT 欠損症	150～300	200～1000	10～20	10～30%	5～20%
家族性 Apo AI-CIII 欠損症 (Schaefer)	111	62	1	ND	3.4
家族性 Apo AI-CIII 欠損症 (Norum)	156	62	6	0.0059	19
Apo AI $_{Milano}$*	208	243	11	13(apo AI+AII)	
Tangier 病	70±20	200±13	2±2	0.65±0.27	2.2±0.6
HDL deficiency with planar xanthomas	260	290	3	2	15
Familial hypoalpha-lipoproteinemia*	165±38	113±29	26±4	NR	NR
Fish-eye disease	207±36	424±97	7±1	38±11	5.3±2.3

mg/dl，NR：not reported　＊ヘテロ接合体

HDL に類似する．HDL 分画には他にアポ AI を含む球状粒子も存在するが，正常 HDL 粒子に比べ粒子径は小さい．LDL 分画には正常より粒子径の小さい LDL 粒子の他に小腸で合成されるアポ B48 を含む multilamelar vesicle が存在し，これはカイロミクロンの水解過程で生じると考えられる．

貧血は赤血球膜の遊離コレステロールとレシチンの増加による溶血性貧血と考えられ，赤血球の形態異常である標的赤血球が出現する．腎障害は糸球体係蹄への脂質蓄積により，遊離コレステロール，レシチンの含量が増加している．角膜混濁は特に周辺に強く小斑点が密に存在する．沈着物の同定はされていないが，電顕的には膜様構造物を含む空胞が多数みられる．腎動脈や大動脈にアテローマ形成がみられるが，早発性冠動脈疾患が生じるかどうかは明らかではない．LCAT 欠損症には完全欠損型と部分欠損型があり，完全欠損型にも抗体交叉蛋白が存在するものがあり heterogeneity がみられる．LCAT 遺伝子は第16染色体に存在し，6つのエクソンからなり440のアミノ酸をコードしている（うち24残基はシグナル・ペプチド）．計算上の分子量は47,090で4カ所の N 結合糖結合部位が存在する[5]．SDS 電気泳動上の分子量は63,000で25% の糖を含む．LCAT cDNA を用いて遺伝子解析が試みられているが，サザンブロットでは異常を見出しえていない[5][6]．

ii）アポ AI 欠損症（familial Apo AI-C III deficiency）

アポ AI 欠損症は1982年 Schaefer[7]と Norum[8]により異なる2家系が見出された．Schaefer らの家系の発端者は42歳の女性で，角膜混濁と重症冠動脈疾患を伴っていた．黄色腫はみられなかった．アポ AI は検出不能でアポ A II も著減していた．興味深いことにアポ C III も欠損していた．家系調査により家系内17名は

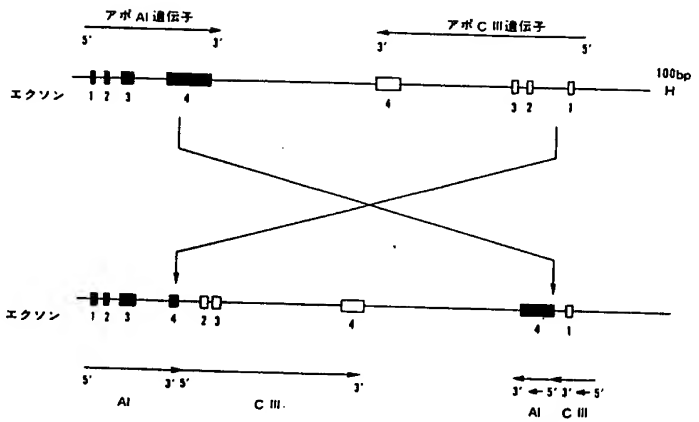

図2　アポAI-C III欠損症における，アポAI，アポC III遺伝子の逆転

アポA I，C IIIが正常の58%，83%に低下しており，うち3名に冠動脈疾患の発症がみられ家族性のアポAI-C III欠損症と考えられた．Norumらの家系の発端者は31歳と33歳の姉妹例でともに角膜混濁と冠動脈疾患を伴っていたが，Schaeferらの報告例と異なり眼瞼，体幹，四肢に黄色腫を認めた．HDLコレステロールは正常の12%に低下し，アポAI，C IIIはともに検出不能，アポA IIは正常の51%に低下していた．また家系内8名はHDLコレステロール，アポAIがそれぞれ正常の67%，59%に低下しておりヘテロ接合体と考えられた．わが国でもHiasaら[9]により同様の症例が報告されている．

アポAI，C IIIの遺伝子はアポA IV遺伝子とともに第11染色体上でclusterを形成している．サザンブロット解析ではSchaeferらの報告例では異常を見出せなかったが，Norumらの報告例ではアポAI-C III遺伝子のrearrangementが認められた．患者のアポAI-C III遺伝子のクローニングにより，患者ではアポAI遺伝子のエクソン4とアポC III遺伝子のイントロン1の間で組換えが起こり，この間の6.0kbのDNAが逆方向に挿入されていることが明らかになった（図2）[10]．このような遺伝子の逆配置は免疫グロブリンやT細胞レセプターのアセンブリの際に生理的に認められ，また病的にはある種のサラセミアのβグロビン遺伝子異常にみられる．この異常遺伝子からのtranscriptの形成はCOS 1細胞への形質導入により認められているが，*in vivo*では発現していないのか，分解を受けやすい蛋白が合成されているのかは明らかでない．

iii）アポAI異常症

1980年Francheshiniら[11]は低HDL血症患者の血清中に等電点電気泳動上正常アポAIの他に，1単位陽性側に泳動される異常アポAIが存在することを見出しアポAI_{Milano}と名付けた．アミノ酸分析によりアポAI_{Milano}は$Arg_{173} \rightarrow Cys$の変異であることが示された[12]．家系内に発端者を含め5名のアポAI_{Milano}のヘテロ接合体がみられ，HDLコレステロールは平均11mg/dl，アポAは平均13mg/dlと低下していたが，角膜混濁や冠動脈疾患はみられなかった．アポAI_{Milano}ヘテロ接合体のアポAIの低下はむしろ正常アポAIの減少が著しいが，この原因は不明である．患者LCAT活性は正常であった．現在までに9種のアポAI異常症が見出されているが，すべてヘテロ接合体で脂質蓄積症状や冠動脈疾患を伴うものはなく，アポAI_{Milano}，アポ$AI_{Marburg}$を除きリポ蛋白代謝異常を伴わない．

遺伝子頻度はアポ$AI_{Marburg}$は3家系で人口1,000：1，他の変異はそれぞれ1家系で人口5,000：1以下で少ない．純化されたアポ$AI_{Marburg}$を用いて

表3　CETA完全欠損症例の血清脂質・アポ蛋白値

	Case1	Case2	Case3	Case4	Case5	正常
T. Chol*	300	340	223	275	341	170±17**
HDL-Chol*	236	231	157	175	281	52±11**
TG*	252	329	77	71	74	86±29**
Apo A-I*	223	258	210	175	232	134±16**
Apo A-II*	51	55	44	34	37	33±4**
Apo B*	67	51	80	67	63	83±16**
Apo C-II*	8.7	9.6	5.4	4.2	10.6	3.0±1.0**
Apo C-III*	34.4	41.2	13.6	15.9	35.3	6.8±1.4**
Apo E*	11.4	19.2	6.2	9.3	21.6	3.5±0.9**

*mg/dl，**Mean±SD

LCAT活性を測定すると，LCATのコファクター作用は正常アポAIの40～60%に低下していた[13]．アポAIのLCAT活性化作用はαヘリックスの構造が重要であると考えられており[14]，アポ$AI_{Marburg}$は1つのコドンが完全に欠損した変異で，蛋白のαヘリックス構造に変化が生じていることがLCAT活性化作用低下の原因と考えられる．

iv）その他のHDL欠損または低下症

a）Tangier病

Tangier病は1961年Frederickson ら[15]により報告された角膜混濁，オレンジ色の扁桃腫大，肝脾腫，リンパ節腫大，末梢神経障害を主症状とする常染色体劣性遺伝疾患で，現在までに20家系23例の報告がある．動脈硬化疾患については，40歳以上の8例のうち3例に冠動脈疾患と2例の脳血管障害の報告がある．HDLコレステロールは正常の4%に低下し，アポA I，A IIは正常の1%，9%に低下しており，特にアポA Iの減少が著しい．本症の原因として，アポIの異化亢進やプロアポA Iから成熟アポA Iへの転換障害が提唱されているが，その遺伝的本態は未だ明らかになっていない．

b）HDL deficiency with planar xanthomas

Lindeakogら[16]が1972年に報告した48歳の女性例で，角膜混濁，肝脾腫に加え，眼瞼や口唇周囲に扁平黄色腫を示し，狭心症を伴っていた．Tangier病と異なり扁桃は正常であった．HDLコレステロール，アポA I，A IIは正常の6%，1%，19%に低下していた．本症の本態は不明である．

c）familial hypoalphalipoproteinemia

1981年Verganiら[17]は低HDL血症と心筋梗塞，突然死が多発する家系を報告した．角膜混濁や黄色腫はみられなかった．HDLコレステロールは正常の51%に減少するが，アポA Iに等電点電気泳動上異常はみられなかった．

d）fish eye disease

1979年Carlsonら[18]は視力障害を伴う強い角膜混濁を伴う2家系を報告した．角膜移植時に摘出した角膜には空胞形成がみられ，遊離コレステロールが増加していた．大動脈弁の石灰化，負荷心電図の虚血性変化を認めた．HDLコレステロールは正常の10%以下に減少し，HDL粒子は径が小さく遊離コレステロールが軽度増加していた．患者HDLにLCATを加えるとHDLのコレステロール・エステルは増加し，粒子径は正常化する．患者LCATは正常HDLの遊離コレステロールをエステル化できないが，VLDL，LDLの遊離コレステロールはエステル化できることから，LCATにはHDLを基質とするαLCATとVLDL，LDLを基質とするβLCATが存在し，fish eye diseaseはαLCAT欠損症であるとする説が提唱されている[19]．

2）HDLが増加する病態

Glueckらは HDLコレステロール値が70～100mg/dlと増加している家系を報告し，この家系では平均寿命が長いことから長寿症候群と呼ばれた．しかし1984年Matsuzawaら[20]はHDLコレステロールが150mg/dl以上に著増し，角膜混濁を伴った2家系を報告した．HDLの代謝過程に障害があり，HDLが増加する病態が存在すると考えられる．

i）コレステロール・エステル転送活性（cholesteryl ester transfer activity：CETA）欠損症

われわれはこれまで5家系のCETA欠損症を見出している．発端者には現在のところ角膜混濁，黄色腫や冠動脈疾患はみられていない．HDLコレステロール値は157～281mg/dlと著しく高く，アポA I，A

表4　FHにおけるLDLレセプターの変異

	症例	前駆体 (KD)	成熟体 (KD)	変異	部位
Class1 合成欠損	FH49	−	−	欠失＞10kb	5′上流域＋エクソン1(A)
	FH26	−	−	欠失～6kb	5′上流域＋エクソン1(A)
	FH381	−	−	欠失5kb	エクソン13～15(C)
	FH651	−	−	欠失4kb	エクソン13, 14(C)
Class2 ゴルジへの輸送障害	FH264	95	95	ナンセンス Cys_{660} Stop	エクソン14(C)
	FH563	120	120	欠失～27bp (不明)	エクソン4(B)
	FH384	135	135		
Class3 LDL結合障害	FH626	100	140	欠失0.8kb	エクソン5(B)
	FH359	100	140	欠失4kb	エクソン7, 8(C)
	KK	115	155	不明	
	FH295	170	210	重複14kb	エクソン2～8(B＋C)
Class4 内部転送障害	FH274	110	150	欠失5.5kb	エクソン16～18(D＋E)
	MN (FH781)	115	155	欠失7.8kb	エクソン16～18(D＋E)
	FH683	115	155	ナンセンス Trp_{792} Stop	エクソン17(E)
	FH763	115	155	フレームシフト	エクソン17(E)
	JD (FH380)	120	160	ミスセンス Tyr_{807} Cys	エクソン17(E)

A：シグナル・ペプチド，B：リガンド結合ドメイン，C：EGF前駆体相同ドメイン，
D：膜貫通ドメイン，E：細胞質ドメイン

II，CIIIに加えアポEの増加を認めた(表3)．HDLの粒子径は増大し，一方LDLの粒子径は小さく不均一な径の分布を示した[31]．CETA欠損症はわれわれの報告した完全欠損例以外に部分欠損例[32][33]が存在し，一様な病態ではないと考えられる．また本症がCETPの欠損や機能異常によるものか，CETPに対する阻害因子が存在するのかは今後の研究課題である．

ii）角膜混濁を伴う高HDL血症

Matsuzawaらが報告した2症例に加え，5例の角膜混濁を有する高HDL血症を経験している．このうち2例は重症の冠動脈疾患を伴っていた．患者ではCETA欠損症と同じくHDL粒子径の増大を認めたが，LDL粒子径は正常であった．ヘパリン静注後のリパーゼ活性では，このうち2例ではLPLは高く肝性リパーゼ活性は著しい低値を示したが，本症のprimary defectは未だ不明である．しかし，これらの症例においては先に述べたHDLを介するコレステロールの逆転送の障害が存在し，組織中のコレステロールの蓄積を防御する機構が円滑に働かないために，脂質蓄積が生じるものと考えられる．

2．LDL代謝異常

1）LDLレセプター異常症 (familial hypercholesterolemia)

LDLレセプター欠損症は，家族性高コレステロール血症(FH)としてよく知られている．結節性黄色腫，角膜輪，早発性冠動脈疾患を主症状とする常染色体優性遺伝疾患で，遺伝子頻度は人口500：1と高い．LDLの異化過程の3分の2はLDLレセプターにより行われ，LDLレセプターを完全に欠くホモ接合体ではLDLの血液中のlife spanは正常の2.5日から6日に延長する．LDLレセプター遺伝子は第19染色体上にあり，833のアミノ酸をコードしている．LDLレセプターは機能的に異なる複数の蛋白ドメインからなる．FHにおけるLDLレセプター異常には多様性があり[34]～[36]種々の構造異常により異なる機能異常が生じることが明らかにされている(表4)．FHの2～6％にはサザンブロット解析によるLDLレセプター遺伝子のrearrangementが認められ，現在までに20種以上の変異が見出されている[34][37]～[43]．LDLを結合したLDLレセプターは細胞内に取り込まれ，シグナル(ステロール結合蛋白と仮定されている)は核内に伝えられ，LDLレセプターやステロール合成の律速酵素であるHMG-CoAレダクターゼの合成を転写レベルで調節する．これにより細胞内コレステロール量のホメオスタシスが保たれている．LDLレセプター遺伝子およびHMG-CoAレダクターゼ遺伝子の5′上流には，GTGまたはGGTGというレプレッサー結合部位が存

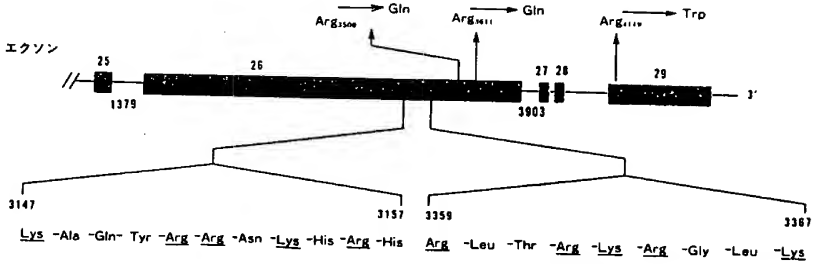

図3　アポBのLDLレセプター結合ドメイン（推定）と変異部位

在する[33][34]．

なおFHの診断は患者の培養線維芽細胞におけるLDLレセプター活性の測定により行われるが，高LDL血症，黄色腫，冠動脈疾患を有し臨床的にFHと診断された多数例でLDLレセプター活性を測定すると，約20％の症例はレセプター活性は正常であり他のLDL代謝異常の存在が示唆される[35]．

2）アポBの異常（図3）

複合型高脂血症は常染色体優性遺伝疾患で家系内にVLDL，LDLのいずれかあるいは両方が増加するものが存在し，同一例でも時期によりVLDLが増加したりLDLが増加したりする．冠動脈疾患の頻度も高い．本症ではアポBの合成亢進が認められるという報告がある[36]が，遺伝的本態は明らかになっていない．アポB遺伝子は第2染色体上にあり，$_{(4563}$～$_{4536)}$のアミノ酸をコードしている[37]．アポB遺伝子の5′上流域にはアポBの転写を正または負に制御する領域が存在するが，アポB合成を抑制する因子は未だ明らかにされておらず，DNAフットプリント法でもこの負の調節領域に結合する蛋白は認められていない[38]．

アポBの3147～3157および3359～3367残基は酸性アミノ酸に富み，特にArg_{3359}～Lys_{3367}はアポEのLDLレセプター結合部位と相同性（Arg-X-X-Arg-Lys-Arg-X-X-Arg/Lys）をもち，レセプター結合部位と考えられている[37]．Grundyらは高LDL血症患者の中に正常者体内で異化を受けにくいLDLを有する患者を見出した[39]．線維芽細胞LDLレセプターへの競合実験により，患者LDLは正常の3分の1に結合親和性が低下していることが示された[40]．このLDLアポBはArg_{3500}→Glnの変異であることが最近明らかにされた．患者血清コレステロール値は247～311mg/dlで同胞3名中72歳と70歳の男性は冠動脈疾患を伴っていたが黄色腫は認められなかった．Ladiasら[41]は87例の冠動脈造影を施行した患者についてアポBのRFLP解析を行い，43歳の女性患者でMspIによる異常断片を認めた．これはArg_{4019}→Trpの変異によることが明らかになった．患者の血清コレステロール値は正常であったがアポBは高値であった．Huangら[42]もMsp Iを用いたRFLP解析により異常パターンを示す例を見出し，Arg_{3611}→Glnの変異であることを明らかにしたが臨床的意義は明らかではない．

おわりに

HDL，LDLの代謝に関与する種々のアポ蛋白，酵素などの異常について記した．LDL，HDLの代謝異常により動脈硬化が促進されることは明らかであるが，LDL代謝系に比べHDLの代謝過程は複雑であり不明な点も少なくない．しかしわれわれが発見したような高HDL血症の病因を明らかにしていくことにより，HDL代謝に関与する因子の分子遺伝学的解明が進むと思われる．

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動脈硬化とアポ蛋白・LDL レセプターの遺伝学

山本 章

血漿リポ蛋白異常は，動脈粥状硬化の主要な危険因子の一つである．血漿リポ蛋白は栄養の影響を受けて強く変動するが，同じ栄養条件下でも高脂血症の発現には大きな個体差がある．血漿リポ蛋白は代謝に影響を及ぼす因子として，リポ蛋白の蛋白質部分（アポリポ蛋白）の分子異常とリポ蛋白レセプターの異常が明らかにされてきた．特に HDL の減少に関連するアポ A-I の分子異常，LDL や中間体の異化の障害につながるアポ B や LDL レセプターの異常，アポ E の異常などを中心として，動脈硬化関連因子の分子遺伝学についての解説を行うことにしたい．

1．リポ蛋白代謝におけるアポリポ蛋白と LDL レセプターの役割

アポリポ蛋白（アポ蛋白）は脂質粒子の表面に付いて，①その両親媒性（水とアブラの双方に親和性をもつ）性質によってリポ蛋白粒子を安定なものとし，②脂質の水解などに関与する酵素の活性を修飾し，また③リポ蛋白の取り込みに働く細胞表面のレセプターへの結合の標的となる，などの役割を果たしている（表1）[1]．これまで十数種のアポ蛋白が同定されているが，そのほとんどの構造と遺伝子は明らかにされ，系統発生の過程も推測されている[1,2]．アポ蛋白の異常は，ある場合は特定のリポ蛋白分画の欠損として現われ，ある場合はリポ蛋白の処理障害のために高脂血症を発現する．その中には動脈硬化に密接に関連するものもあり，逆に動脈硬化になりにくいもの，また他の合併症，例えば膵炎を起こすものなどがある[3,4]．

〔キーワード＆略語〕
リポ蛋白（lipoproteins）
アポリポ蛋白（apolipoproteins）
LDL レセプター（LDL receptor）
動脈硬化（atherosclerosis）
家族性高コレステロール血症：familial hypercholesterolemia（FH）
高脂血症（hyperlipidemia）
アポ E 同位体（apo E isoforms）

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リポ蛋白代謝に関係するレセプターには，LDL レセプター，マクロファージのもつ scavenger レセプター，肝細胞にあると考えられている remnant レセプター，HDL の代謝に関係する HDL レセプターがあるが，そのうち実体が完全に明らかにされているのは LDL レセプターのみである．LDL レセプターは肝臓で合成・分泌されるトリグリセライド・rich リポ蛋白である超低密度リポ蛋白（very low density lipoprotein：VLDL）がリポ蛋白リパーゼの作用によってトリグリセライドの大半を失い（図1），さらに肝臓のトリグリセライドリパーゼの作用を受け修飾されてできた代謝産物，低密度リポ蛋白（low density lipoprotein：LDL）の異化に働く細胞表面小器官であって，ほぼすべての組織細胞に広く分布している．その欠損症は典型的な高脂血症の一種である家族性高コレステロール血症（familial hypercholesterolemia：FH）を起こす[5]．以下，特に動脈硬化に関連してそのマーカーとなるアポ蛋白異常と FH について解説する．

2．アポ蛋白異常と動脈硬化

1）アポ A-I 異常症

1961年最初の患者が発見された地名から Tangier 病と呼ばれる疾患がある[6]．これはコレステロールの沈着のために腫大した扁桃，角膜の小点状混濁と多発性神経炎を主徴とするほか，動脈硬化性疾患の合併も